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Effect of gamma radiation on sodium channels in different conformations in neuroblastoma cells

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We studied the dose-response relationship between gamma radiation and batrachotoxin-stimulated sodium influx in neuroblastoma cells in tissue culture. We also tested the hypothesis that changes in sodium channel conformation may alter the radiosensitivity of the channel. We found that gamma radiation inhibited toxin-stimulated ^{22}Na uptake at doses beyond a threshold of 200–300 Gy. No effects were seen following doses below 100 Gy. This inhibition of sodium permeability was seen when the cells were irradiated with sodium channels in the closed or inactivated, nonconducting states. However, when the channels were in the toxin-opened, conducting state, gamma radiation had no effect at doses up to 2000 Gy. Our results support earlier electrophysiological studies that showed that high doses of ionizing radiation are required to produce a measureable decrease in sodium permeability. In addition, our data suggest that by changing the sodium channel conformation, batrachotoxin appears to alter radiosensitive chemical bonds in the gating or ion-conducting portion of the channel.

Introduction

Both chemical and physical agents can be useful in demonstrating structural and functional properties of biological membrane macromolecules. In differentiated post-mitotic cells, such as neurons, alterations of proteins by ionizing radiation may have greater immediate functional consequences than does disruption of nucleic acids. Enzymes vary considerably in the extent to which they are inactivated by ionizing radiation [1], and other protein species are likely to show a similar spectrum of radiosensitivity. A large body of biophysical data exists showing that voltage-sensitive sodium channels are inactivated by ultraviolet radiation at doses that do not affect potassium channel function [2–4]. One might expect that ionizing radiation, comprising a higher energy spectrum than does ultraviolet radiation, may interact with integral membrane proteins in a different way than does ultraviolet radiation. Few studies, however, have used cellular biophysical techniques to study the effects of ionizing radiation on neuronal ion channels. Schwarz and Fox [5] reported that monochromatic X-rays, at doses in excess of 100 Gy (1 Gy = 100 rads), selectively reduce sodium currents in isolated frog sciatic nerves under voltage-clamp. At no dose did they

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

find an effect that could underlie neuronal excitation earlier reported as a consequence of ionizing radiation (reviewed in Ref. 6). They suggested that earlier studies reporting an increase in excitability in isolated nerve preparations might be explained by injury currents. Wixon and Hunt [7], studying sodium fluxes in isolated rat brain synaptosomes, found that high-energy electrons decreased sodium uptake beginning at doses as low as 0.5 Gy.

The purpose of these studies was two-fold. First, we wished to establish a dose-response relationship between gamma radiation and sodium influx through voltage-dependent channels to see if the radiosensitivity reported by Wixon and Hunt [7] is present in a whole-cell preparation. Second, we investigated whether the conformational state of the channel could influence its radiosensitivity.

Materials and Methods

Tissue culture

Cells from the neuroblastoma clone N18 were grown in Dulbecco's modified Eagle medium (GIBCO) supplemented with fetal bovine serum (10% v/v). The cells were seeded into 24-well cluster trays and, after 1 or 2 days, were fed medium plus 0.5% fetal bovine serum (GIBCO) and 1 mM dibutyryl cAMP (Sigma). Without this latter treatment, cells grew rapidly to confluency and began to slough from the well bottoms. In the presence of dibutyryl cAMP, a drug which induces electrophysiological and morphological differentiation [8], the cells divided more slowly and adhered well to the trays. Cells were then used for experiments after growing in the presence of dibutyryl cAMP for 3–4 days, at which time they had formed a confluent, adherent monolayer.

Irradiation

N18 cells in the cluster trays were washed three times with Dulbecco's phosphate-buffered saline (GIBCO, pH 7.3) (normal saline) and allowed to incubate in either normal saline or other test solutions (described in Results) for 30 min prior to and during irradiation. Irradiation was done in the cobalt-60 facility of the Armed Forces Radiobiology Research Institute at constant dose rates whenever possible. For wide dose ranges that included doses below 100 Gy, two different dose rates were required. The facility contains approx. 100000 Ci of ^{60}Co in 104 separate elements. Unilateral dose rates of 0.01–25 Gy/min and bilateral dose rates of 0.08–57 Gy/min can be administered with error bounds of ±5%. Dosimetry was done using tissue-equivalent ion chambers and thermoluminescent dosimeters within the wells of the plastic trays.

Sodium flux assay

We used, with slight modifications, the methods developed by Catterall [9,10]. Cells were incubated for 60 min at 26°C in toxin-incubation medium comprising 135.4 mM KCl, 50 mM Hepes-Tris (pH 7.4), 5.5 mM glucose, 0.8 mM MgSO, 1 g/l bovine serum albumin, and various concentrations of batrachotoxin (kindly supplied by Dr. John W. Daly, NIAMDD, National Institutes of Health, Bethesda, MD). This medium allows the toxin to reach equilibrium with the receptor site without altering ion gradients. The toxin-containing medium was removed and the cells were rinsed twice in 15 s using a standard medium consisting of 5.4 mM KCl, 130 mM *N*-methyl-D-glucamine, 50 mM Hepes (pH 7.4), 5.5 mM glucose and 0.8 mM $MgSO_4$. The cells were then incubated for 30 s in assay medium containing 5.4 mM KCl, 120 mM *N*-methylglucamine, 10 mM NaCl, 5 mM ouabain, 50 mM Hepes, 5.5 mM glucose, 0.8 mM $MgSO_4$ and 1 μCi/ml $^{22}NaCl$. Because of the very slow dissociation rate of batrachotoxin, it was not necessary to include the toxin in the assay medium [11]. Under these conditions the initial rates are measured with the sodium pump inhibited and the membrane potential held constant. The uptake was then terminated by washing the cells three times with a stop solution containing 163 mM *N*-methyl-D-glucamine, 5 mM Hepes, 5.5 mM glucose, 0.8 mM $MgSO_4$ 1.8 mM $CaCl_2$ and 3 μM tetrodotoxin (Sigma). This medium allows the extracellular ^{22}Na to be removed under conditions that stop influx and prevent efflux of ^{22}Na. The cells were then solubilized in 0.5% Triton X-100 in water, and the radioactivity of the solubilized cell samples was determined in a liquid scintillation counter.

Proteins were determined from three separate wells per tray using the Bio-Rad protein assay.

Our choice of 30 s uptake assay time was justified by initial experiments that confirmed that the rate of ^{22}Na influx remained linear for more than 2 min. Nonspecific Na uptake was measured as the uptake in cells without batrachotoxin. This was not significantly different from the uptake in 3 μM tetrodotoxin, without batrachotoxin. The rationale for using the above solutions has been reviewed by Catterall [9].

Results

Batrachotoxin-stimulated Na influx

The influx of sodium that resulted from activation of the sodium channels by batrachotoxin was reproducible from experiment to experiment and agreed with the results published by Catterall [9,12]. Fig. 1 shows the effect of batrachotoxin on initial rates of ^{22}Na uptake in unirradiated cells. The data were pooled from three different experiments performed during a 3-month period. Each experiment was normalized to the response obtained in the presence of 1 μM batrachotoxin. Since the rate of ^{22}Na influx is directly proportional to the fraction of sodium channels activated by batrachotoxin, as shown by Catterall [12], the relationship between the rate of uptake and toxin concentration can be described by a modified Michaelis-Menten equation of the form:

$$v = V_{max}(1 + K_m/x)^{-1},$$

where v is the rate of ^{22}Na uptake at various toxin concentrations (x) and is linearly proportional to the fraction of activated sodium channels; V_{max} is the maximum rate of ^{22}Na uptake at infinite toxin concentration; and K_m is the toxin concentration at half-maximum uptake and reflects the degree of binding or affinity of the toxin to its binding site. The pooled data in Fig. 1 were well fit by this equation; the calculated values of V_{max} and K_m were 127 nmol/mg protein per min and 0.54 μM, respectively. This toxin dose-response relationship is quantitatively similar to that reported by Catterall [9,12].

Radiation dose-response relationship

There was considerable variability from experiment to experiment in the degree to which sodium influx was inhibited by gamma irradiation. Such experiment-to-experiment variability in the effects of ionizing radiation has been noted by other investigators (e.g., Ref. 13), indicating the importance of using each set of cultures as its own non-irradiated control. Within a given set of cultures used for any one experiment, results from triplicate sets of wells were consistent. No effect of radiation was found at doses below 100 Gy. Consistent reduction in maximum Na influx was seen only after doses above 200 Gy. Fig. 2 shows the results of 10 separate experiments, done on different cultures on different days, from which the data have been pooled. Within each experiment, for a given condition, measurements from three separate wells were averaged. In order to pool these averages, data from a range of radiation doses were combined. This was because all experiments did not share the same radiation dose sequence. Thus, for example, data from radiation doses between 200 and 600 Gy were combined. In these experiments the uptake induced by 1 μM batrachotoxin was compared for various radiation doses from 100 to 3000 Gy. Uptake at doses below 200 Gy was not significantly different from

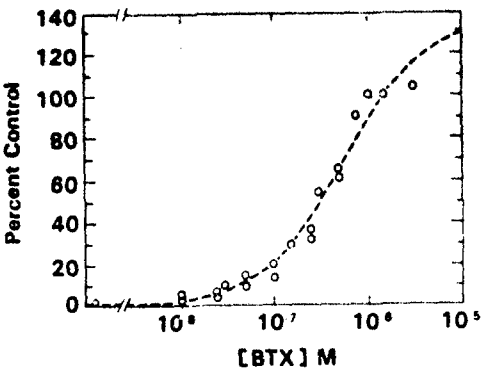

Fig. 1. Dose-response relationship between batrachotoxin concentration and ^{22}Na uptake. Data were pooled from three separate experiments. The data were normalized as a percent of the response obtained with 1 μM batrachotoxin in each experiment (this point is termed control). Each point is the average of measurements from three separate wells at that batrachotoxin dose. The curves were fitted to a modified Michaelis-Menten equation using a computer-generated least-squares method ($R^2 = 0.98$). The calculated values of the parameters V_{max} and K_m were 127 nmol/mg protein per min and 0.54 μM, respectively (mean value of v at 1 μM batrachotoxin was 91 nmol/mg protein per min; V_{max}, as a percent of the control value, was 137).

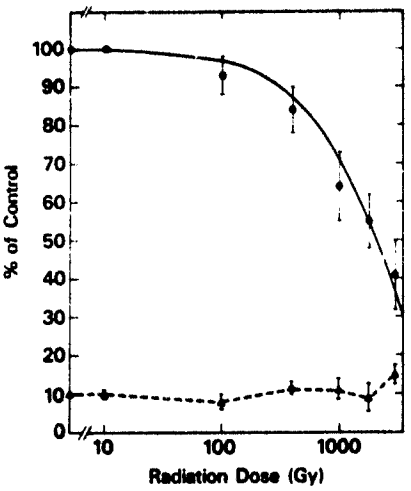

Fig. 2. Dose-response relationsohip between gamma-radiation and ^{22}Na uptake. Both the batrachotoxin-stimulated (●) and the non-specific (▲) ^{22}Na influx at various radiation dose ranges are shown. For batrachotoxin-stimulated flux, data were normalized and pooled from 10 different experiments in the manner described in Fig. 1. In addition, responses obtained over a range of radiation doses were pooled as follows (dose range and number of experimental averages pooled): 0 Gy, $n = 9$; 10–100 Gy, $n = 4$; 200–600 Gy, $n = 10$; 800–1200 Gy, $n = 7$; 1600–2000 Gy, $n = 5$; 2800–3200 Gy, $n = 5$. The non-specific uptake, pooled as above (for above dose ranges: $n = 9$, 5, 18, 8, 4, 4, respectively), was not normalized and is shown as initial rate of uptake. Each point represents the mean and standard error of the mean of the average uptakes pooled over the indicated dose ranges. The curve for the batrachotoxin-stimulated uptake data is a non-linear least-squares fit to the equation $y = 100\ e^{(-x/k)}$ where k was computed as 2900 Gy ($R^2 = 0.99$). The radiation dose rates for these and all experiments shown in subsequent figures were similar; between 66 and 67 Gy/min.

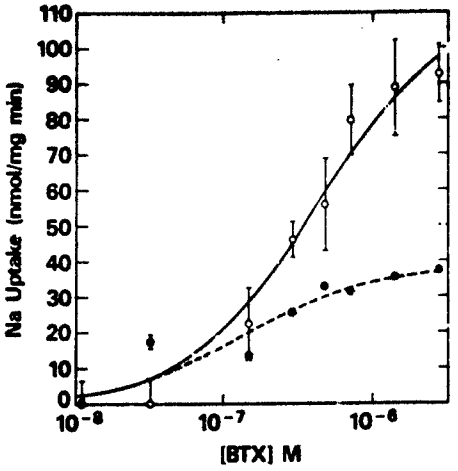

Fig. 3. Dose-response relationship between batrachotoxin concentration and ^{22}Na influx in irradiated cells (●) and non-irradiated controls (○). Each data point represents the mean and standard error of the mean of measurements from three different wells for each batrachotoxin dose at each radiation dose (0 and 1500 Gy). The curves were fit by the equation described in Fig. 1 ($R^2 > 0.9$ for each curve). The calculated values of the parameters V_{max} and K_m were respectively: 112 nmol/mg protein per min and 0.45 μM for the control curve; 38 nmol/mg protein per min and 0.14 μM for the data from irradiated cells.

control uptake. Thereafter, there was a progressive fall in Na influx as an exponential function of increasing doses of gamma radiation. As shown also in Fig. 2, there was no significant increase in the nonspecific (leak) influx of ^{22}Na at doses up to 3000 Gy.

It is possible that radiation may alter the affinity of batrachotoxin for the sodium channel. If so, then the apparent inhibition of ^{22}Na uptake could simply reflect this loss of toxin affinity rather than an effect on the channel. To assess this possibility we examined the dose-response relationship between batrachotoxin and ^{22}Na uptake at various radiation doses. As shown in the experiment of Fig. 3, the predominant effect of irradiation was a depression of the maximum rate of sodium uptake (V_{max}). The apparent affinity (reflected by K_m) of batrachotoxin for its receptor was not reduced; in fact, the curves were consistently shifted to the left.

Effect of channel conformation on radiosensitivity

To study the effect of channel conformation on radiosensitivity, N18 cells were irradiated under three conditions. In one group, the cells were irradiated in normal saline. Under this condition, the transmembrane potential is sufficiently negative so that as many as 20% of the sodium channels are in the closed state [14,15]; the rest are inactivated. Cells in the second group were irradiated in high K^+-saline (the toxin-incubation medium without batrachotoxin). Under this condition, the cells are depolarized to close to 0 mV transmembrane potential and virtually all the channels are in the inactivated state. The third group was irradiated in the presence of batrachotoxin in the toxin-incubation medium (high K^+). Most of the channels under this condition are activated and remain in the toxin-mod-

ified open state [11,16]. In addition, in some experiments cells were incubated in the presence of 50 μM *N*-bromoacetamide to remove channel inactivation. The drug acts at a different site than does batrachotoxin and inhibits fast inactivation with little effect on activation [17,18]. That *N*-bromoacetamide had an effect from the outside of the membrane was shown in preliminary experiments showing a 40% greater maximum uptake achieved with *N*-bromoacetamide plus batrachotoxin compared with uptake in the presence of batrachotoxin alone. The experiments were done as described in Materials and Methods except that during the 30 min prior to and during irradiation the cells were incubated in either normal saline, high K^+-saline, toxin-containing medium with or without *N*-bromoacetamide, or *N*-bromoacetamide in normal saline. Immediately following irradiation, for those cultures not containing batrachotoxin, the uptake assay was carried out as outlined in Methods. For the cultures that contained toxin during irradiation, the toxin-incubation step was omitted, and the uptake assay was begun immediately following irradiation by washing with the standard medium and proceeding as described in Methods. Thus, for all groups, the toxin-incubation periods were the same.

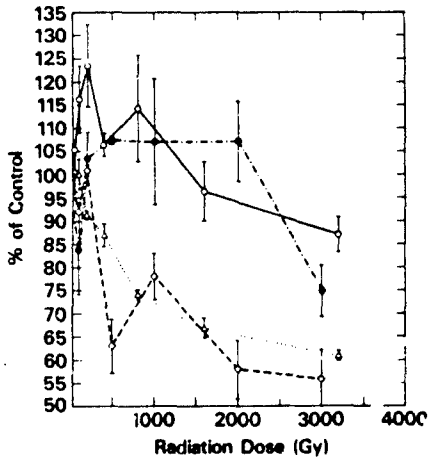

Fig. 4. Dose-response relationship between radiation dose and ^{22}Na uptake under different incubation conditions. Data were obtained from two separate experiments. Within each experiment cells were irradiated either in the presence of batrachotoxin, 1 μM, in toxin-incubation medium (○, ●), in toxin-incubation medium (high K^+) without batrachotoxin (△, ◇), or in normal saline (not shown). After irradiation, the latter two groups of cells were incubated for 60 min in 1 μM batrachotoxin before uptake assay, as described in Methods. In order to compare the results obtained under these different conditions, data from each group were normalized as a percent of the uptake in the absence of radiation.

The effect of radiation on cells with sodium channels in the different conformational states is shown in Fig. 4. The cells in wells containing toxin-free normal saline (data not shown) or toxin-free high K^+-saline showed the typical dose-dependent inhibition of ^{22}Na influx beginning at doses of about 200 Gy. In contrast, the cells in wells containing batrachotoxin during irradiation were remarkably resistant to this effect. No significant inhibition of ^{22}Na influx was seen up to doses in excess of 2000 Gy. Addition of *N*-bromoacetamide to normal saline during irradiation did not alter the radiosensitivity, and when *N*-bromoacetamide was added to batrachotoxin during irradiation, there was no change in the protection afforded by batrachotoxin alone.

Discussion

Our results agree with earlier studies that concluded that ionizing radiation causes reduction in neuronal excitability by blocking voltage-sensitive sodium channels. The dose required to reach a threshold effect was of the same order of magnitude as that found using electrophysiological techniques (reviewed in Ref. 6). Thus, Gaffey [19] found that 1600–2000 Gy of 200-kV X-rays were required to attenuate the amplitude of maximal action potentials in isolated frog sciatic nerves. The duration of the compound action potential, however, increased beginning at doses of 100–200 Gy. In the same preparation under voltage clamp, Schwarz and Fox [5] found that a threshold dose of 80 Gy of monochromatic and 100 Gy of continuous-spectrum X-irradiation was required before peak sodium currents were decreased. We could not confirm the results of Wixon and Hunt [7], who found that high-energy electrons decreased the veratridine-stimulated uptake of sodium in rat brain synaptosomes at doses as low as 0.5 Gy. Perhaps this difference in radiosensitivity can be explained by an absence of cellular protective and repair mechanisms in synaptosomes.

What may explain the striking degree of radioprotection afforded by the presence of

batrachotoxin during irradiation? It is unlikely that such a small amount of toxin could have a significant 'quenching' effect. It is possible that irradiation prevents the binding of batrachotoxin to the sodium channel complex. If so, then these experiments would suggest that bound toxin confers protection against subsequent irradiation. We believe, however, that the reduction in sodium uptake is not due to effects on toxin binding. If binding were reduced but not completely blocked, there might be measurable alteration in the affinity of the toxin for its binding site. However, from dose-response curves of batrachotoxin versus ^{22}Na uptake at different radiation doses, such as those of Fig. 3, we found that the maximum rate of ^{22}Na uptake (V_{max}) was affected to a much greater extent than was the affinity of batrachotoxin for its binding site (where affinity is inversely related to the parameter (K_m). Furthermore, radiation slightly lowered the value of K_m, thereby suggesting a slight increase rather than a decrease in the affinity of the toxin toward its binding site. If radiation were to prevent the binding of batrachotoxin in an all-or-none manner, there would be a decrease in V_{max} indistinguishable from that caused by disruption of the ion-conduction subunits of the sodium channels; but direct measurement of sodium current by voltage-clamp, for which batrachotoxin is not required, showed that radiation specifically inhibits the sodium conductance [5]. We therefore hypothesize that during the conformational change induced by batrachotoxin, certain radiosensitive chemical bonds undergo a transition to a more radioresistant state. In voltage-clamp studies of the effect of ultraviolet radiation on sodium channels of frog nodes of Ranvier, Hof and Fox [20] obtained results similar to our observations. Sodium channels appeared least sensitive to ultraviolet light flashes applied shortly after a depolarizing step from a hyperpolarized prepotential. At that time, most of the sodium channels were in the open, non-inactivated state.

Although specific molecules, such as tyrosine and tryptophan, appear to be involved in ultraviolet photoreaction of the voltage-dependent sodium channel [21], there have been no studies to implicate particular molecular species in the interaction of ionizing radiation with the channel. The inhibition of sodium conductance by ultraviolet radiation appears sufficiently different from the process by which ionizing radiation interacts with the channel that extrapolation is not possible. For example, Schwarz and Fox [5] found that blocking of sodium conductance by ionizing radiation occurred after a delay of about 10 min and after a threshold dose of 80 Gy. In contrast, the effect of ultraviolet radiation had no threshold and was of immediate onset. This suggests that whereas ultraviolet radiation reacts directly with sensitive molecules of the sodium channel [2–4], ionizing radiation may indirectly react with the channel through multiple photon interactions or the formation of free radicals [5].

Further studies of the radiosensitivity of the sodium channel may lead to an understanding of which chemical structures are involved in the loss of function and may provide additional knowledge of the nature of sodium channel structure-activity relationships.

Acknowledgements

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